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Chimeric adenovirus type 5/35 vector encoding SIV *gag* and HIV *env* genes affords protective immunity against the simian/human immunodeficiency virus in monkeys

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Abstract

Replication-defective adenovirus type 5 (Ad5) vector-based vaccines are widely known to induce strong immunity against immunodeficiency viruses. To exploit this immunogenicity while overcoming the potential problem of preexisting immunity against human adenoviruses type 5, we developed a recombinant chimeric adenovirus type 5 with type 35 fiber vector (rAd5/35). We initially produced a simian immunodeficiency virus (SIV) *gag* DNA plasmid (rDNA-Gag), a human immunodeficiency virus type 1 (HIV-1) 89.6 *env* DNA plasmid (rDNA-Env) and a recombinant Ad5/35 vector encoding the SIV *gag* and HIV *env* gene (rAd5/35-Gag and rAd5/35-Env). Prime-boost vaccination with rDNA-Gag and -Env followed by high doses of rAd5/35-Gag and -Env elicited higher levels of cellular immune responses than did rDNAs or rAd5/35s alone. When challenged with a pathogenic simian human immunodeficiency virus (SHIV), animals receiving a prime-boost regimen or rAd5/35s alone maintained a higher number of $CD4^+$ T cells and remarkably suppressed plasma viral RNA loads. These findings suggest the clinical promise of an rAd5/35 vector-based vaccine.

Keywords: DNA vaccine; Recombinant Ad5/35; Cellular immunity; Pathogenic SHIV challenge

Introduction

HIV-specific cellular and humoral responses play a critical role in controlling viral replication and disease progression (Mascola, 2003; Letvin et al., 2002; Musey et al., 1997). It has been reported that recombinant live virus- and bacteria-based vaccines such as recombinant adenovirus (Mascola et al., 2005; Shiver et al., 2002) recombinant poxvirus (Someya et al., 2004; Amara et al., 2001), and recombinant BCG (Ami et al., 2005; Someya et al., 2005) elicited high levels of effective immunity against immunodeficiency viruses when used either alone or in conjunction with other vectors.

Adenoviruses, which are associated with benign pathologies in humans, are attractive for use in HIV vaccines because their genome has been extensively studied and because methods for constructing recombinant vectors with them are well established (Imler, 1995). A regimen that primes with DNA and then boosts with rAd5 is known to protect macaques against SHIV challenge by inducing high levels of viral-specific immunities (Shiver et al., 2002). However, Ad5 has been prevented from fully realizing its clinical potential (Catanzaro et al., 2006) because of preexisting humoral immunity to adenoviruses (Barouch et al., 2004; Casimiro et al., 2003). A previous study demonstrated that neutralizing antibodies against Ad5 are widespread in healthy blood donors in the developed world, whereas neutralizing

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antibodies against Ad35 are rare (Kostense et al., 2004; Vogels et al., 2003). Furthermore, patients at risk of AIDS were reported to have a much higher seroprevalence of Ad5 than Ad35 (Kostense et al., 2004).

We have recently constructed an adenovirus serotype 5 vector that possesses serotype 35 fiber (rAd5/35) that encodes the simian immunodeficiency virus (SIV) *gag* gene or human immunodeficiency virus type 1 (HIV-1) IIIB *env* gp160 gene (Xin et al., 2005). The rAd5/35 vector, which lacks the E1 and E3 genes that are responsible for viral replication (Imler, 1995), interacts with CD46-expressing cells (Gaggar et al., 2003) and antigen-presenting cells (Rea et al., 2001). The rAd5/35 vector was shown to enter human dendritic cells (DC) more efficiently than Ad5 and activate T cells *ex vivo* (Ophorst et al., 2004), suggesting that a rAd5/35 vector-based vaccine may be an effective activator for T cell immunity. However, rAd5/35 vector-expressing measles virus hemagglutinin was less immunogenic than rAd5 in low-dose immunization in macaques and suggested that fiber exchange may not circumvent anti-Ad5 immunity during acute Ad5 infection in mice (Ophorst et al., 2004).

Recently, hyper variable regions on the Ad5 hexon protein were successfully replaced with those of the rare Ad48, allowing preexisting anti-vector immunity to be circumvented (Roberts et al., 2006). We previously showed that, when combined with plasmid DNA, the rAd5/35 vector elicited high levels of cellular responses that in turn protected mice from challenge with a virulent vaccinia virus encoding HIV-1 BH8 env (Xin et al., 2005). Furthermore, the rAd5/35 vector-based vaccine was not affected by the preexisting anti-Ad5 immunity in mice.

These results suggest that a vaccine combining plasmid DNA and the rAd5/35 vector may induce effective viral-specific immunities in macaques. In this study, we determined the vaccine efficacy of relatively high doses of rAd5/35 vector-based HIV vaccine in an SHIV macaque model.

Results

Vaccine-induced T cell responses to SIV Gag and HIV Env

After priming with rDNA-Gag and rDNA-Env, Gag- and Env-specific IFN-γ spot-forming cells (SFC) were detected in both the rDNA and the prime-boost groups (Fig. 1A). The numbers of SFC against Gag peaked at 16 weeks in the rDNA and the prime-boost groups, with numbers averaging 120 in the rDNA group and 128 in the prime-boost group. ELISPOT responses to Env also peaked at 16 weeks, with average numbers of 33 in the rDNA group and of 66 in the prime-boost group. Gag- and Env-specific ELISPOT responses were not observed in animals immunized with cDNA (SFC ≤ 10).

Two serial high-dose injections with rAd5/35-Gag and rAd5/35-Env per animal dramatically augmented Gag- and Env-specific ELISPOT responses in the prime-boost group (Fig. 1A). At Week 150, the prime-boost group averaged 53 Gag-specific and 35 Env-specific SFC. Two weeks after the two serial injections with rAd5/35 (154 weeks), the average number of Gag-specific SFC increased to 1073 and that of Env-specific SFC to 340. Two serial injections with rAd5/35s also elicited higher ELISPOT responses in the rAd5/35 group (Fig. 1A), with the average number of Gag-specific SFC increasing from 35 to 570 and that of Env-specific SFC, from 15 to 165. The levels of both Gag and Env ELISPOT responses at the time of virus challenge (154 weeks) significantly increased in the prime-boost group, and they were higher than those in the rDNA (Gag, $p<0.01$; ENV, $p<0.01$) and the cDNA (Gag, $p<0.01$; Env, $p<0.01$) groups. Serial injections with cAd5/35s failed to enhance responses to Gag or Env in either the rDNA or the cDNA group.

Vaccine-induced antibody responses to SIV Gag and HIV Env

Both anti-Gag- and Env-specific IgG responses were detected after serial inoculations with rDNA-Gag and rDNA-Env (Fig. 1B). Anti-Gag-specific IgG titers peaked at 16 weeks in the rDNA group, averaging 123 and at 32 weeks in the prime-boost group, averaging 155 (Fig. 1B, upper panel). The anti-Env-specific IgG titers for both the rDNA and the prime-boost groups peaked at 32 weeks, with titers of 200 and 183, respectively (Fig. 1B, lower panel). Two serial injections with rAd5/35-Gag and rAd5/35-Env to the prime-boost group enhanced both anti-Gag- and anti-Env-specific IgG titers, with the former increasing from an average of 100 at 150 weeks to an average of 823 at 154 weeks and the latter increasing from an average of 74 at 150 weeks to 2240 at 154 weeks. Two serial injections with rAd5/35-Gag and rAd5/35-Env to the rAd5/35 group generated both anti-Gag- and anti-Env-specific IgG responses, with the peak titers in the former averaging 335 and in the latter, 480. Both the anti-Gag- and Env-specific IgG titers at the time of virus challenge (154 weeks) in the prime-boost group were significantly higher than those in the rDNA (Gag, $p<0.01$; Env, $p<0.01$) and the cDNA (Gag, $p<0.01$; Env, $p<0.01$) groups. When anti-HIV-89.6-specific neutralization antibody responses were measured at 154 weeks using the purified serum IgG, the rDNA, the rAd35 and the prime-boost groups showed an average neutralization of 4%, 10% and 24% respectively (data not shown), suggesting that the antibody may not contribute to the partial protection from virus challenge in monkeys. Two serial injections with Ad5/35-LacZ had no effect on anti-Gag and anti-Env IgG responses.

Plasma viral RNA loads and $CD4^+$ T cell counts after challenge with SHIV

To evaluate the efficacy of a prime-boost vaccine regimen, all animals received an intravenous challenge with 20 $TCID_{50}$ of highly pathogenic SHIV at 154 weeks (Table 1). Peak plasma RNA loads (Fig. 2A) and rapid or transient $CD4^+$ T cell loss (Fig. 2B) were observed within 2 weeks after challenge (acute phase). The cDNA group had the greatest viral RNA loads (peaking at an average of 4.5×10^9 copies/ml) and experienced dramatic loss of $CD4^+$ T cells (≤ 50 cells/μl). Although its peak viral RNA loads (an average of 4.9×10^8 copies/ml) were almost ten-fold less than those of the cDNA group ($p<0.01$), the rDNA group likewise showed reduced $CD4^+$ T cell numbers

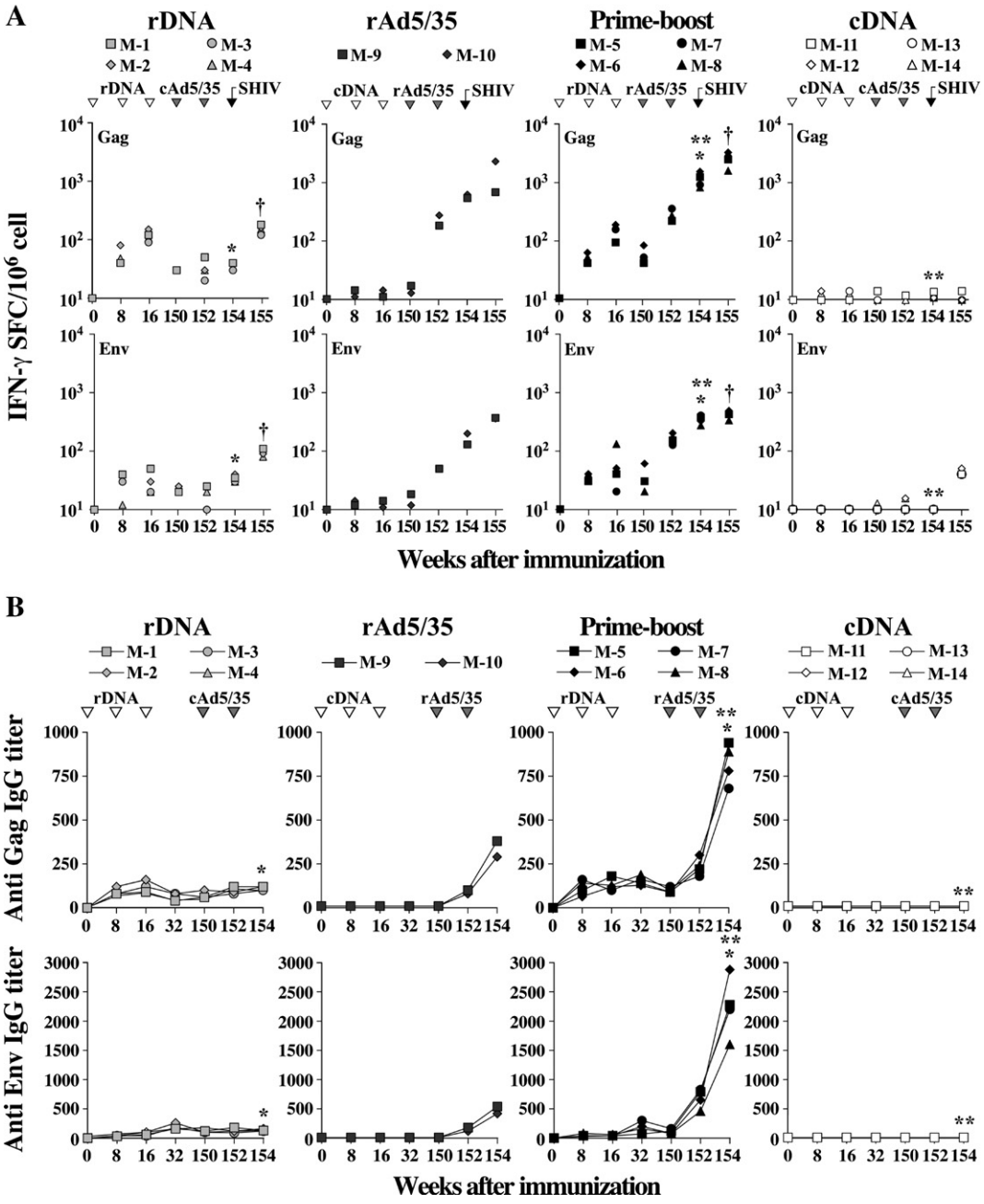

Fig. 1. Cellular and humoral responses of vaccinated animals. (A) Freshly isolated PBMC were stimulated with SIV Gag and HIV Env peptides. An ELISPOT assay was used to monitor the numbers of antigen-specific IFN-γ spot-forming cells. *rDNA vs. prime-boost, $p<0.01$ (154 weeks). **Prime-boost vs. cDNA, $p<0.01$ (154 weeks). †rDNA vs. prime-boost group, $p<0.01$ (155 weeks). (B) End-point Gag- and Env-specific plasma IgG titers were determined in the course of immunization using ELISA. *rDNA vs. prime-boost, $p<0.01$ (154 weeks). **Prime-boost vs. cDNA, $p<0.01$ (154 weeks).

(≤40 cells/μl, no significance). The prime-boost group showed greater than 600-fold reduction in peak viral loads and maintained higher $CD4^+$ T cell numbers compared to the cDNA group (the peak viral loads of the prime-boost group in the acute viral phase: an average of 7.3×10^6 copies/ml, $p<0.01$; $CD4^+$ T cell numbers of the prime-boost group: an average of 616 cells/μl, $p<0.01$). The initial viremia and $CD4^+$ T cell numbers in the prime-boost group were also significantly lower than those in the rDNA group (peak viral RNA loads, $p<0.01$; $CD4^+$ T cell numbers, $p<0.01$). The rAd5/35 group showed 600-fold reduction in the peak viral loads (an average of 5.3×10^6 copies/ml) compared to the cDNA group and maintained higher $CD4^+$ T cell numbers (an average of 500 cells/μl).

During the set-point phase, the cDNA group showed higher viral RNA loads ($\geq5\times10^6$ copies/ml) and lower $CD4^+$ T cell numbers (≤20 cells/μl) (Fig. 2). The set-point viral RNA loads of the rDNA group (averaging 10^6 copies/ml) were approximately five-fold lower than those of the control group, but the $CD4^+$ T cell numbers recovered by no more than 100 cells/μl. Two animals (M-5 and M-6) of the prime-boost group showed well-controlled set-point viral loads and minimal $CD4^+$ T cells

Table 1
Immunization and challenge schedule

Group (regimen)	Monkey no.	Priming immunization and route	Schedule (week of priming)	Booster immunization and route	Schedule (week of boosting)	SHIV challenge
rDNA	M-1, M-2, M-3, M-4	rDNA-Gag, 2.5 mg, i.m. and rDNA-Env, 2.5 mg, i.m.	0, 8, 16	cAd5/35, 2×10^{11} vp, i.d.	150, 152	20 $TCID_{50}$, i.v., Week 154
Prime-boost	M-5, M-6, M-7, M-8	rDNA-Gag, 2.5 mg, i.m. and rDNA-Env, 2.5 mg, i.m.	0, 8, 16	rAD3/35-Gag, 10^{11} vp, i.d. and rAD3/35-Env, 10^{11} vp, i.d.	150, 152	20 $TCID_{50}$, i.v., Week 154
rAd5/35	M-9, M-10	cDNA, 5 mg, i.m.	0. 8, 16	rAD3/35-Gag, 10^{11} vp, i.d. and rAD3/35-Env, 10^{11} vp, i.d.	150, 152	20 $TCID_{50}$, i.v., Week 154
cDNA	M-11, M-12, M-13, M-14	cDNA, 5 mg, i.m.	0. 8, 16	cAd5/35, 2×10^{11} vp, i.d.	150, 152	20 $TCID_{50}$, i.v., Week 154

loss, while the other two macaques (M-7 and M-8) of the same group showed slightly higher viral RNA loads and lower $CD4^+$ T cell numbers. The set-point viral RNA loads for M-6 were within the detection limit (500 copies/ml) and those for M-5 ranged from <500 to 3×10^3 copies/ml. The viremia-controlled M-6 showed more than 800 $CD4^+$ T cells/μl and M-5 showed 600 cells/μl, with the exception of the transient loss (about 350 cells/μl) occurring at 6 and 18 weeks. Set-point viral RNA loads for both M-7 and M-8 ranged from 3.7×10^3 to 4.2×10^4 copies/ml, while $CD4^+$ T cell numbers ranged from 300 to 600 cells/μl. Of the two macaques in the rAd5/35 group, one showed enhanced protection, while the other did not. M-9 suppressed viral loads to less than 10^4 copies/ml and maintained $CD4^+$ T cell numbers at levels of more than 500 cells/μl. The viral loads for M-10 ranged from 10^5 to 10^6 copies/ml, and its $CD4^+$ T cell numbers were less than 300 cells/μl. Throughout the set-point phase, average plasma viral RNA loads and $CD4^+$ T cell numbers in the prime-boost group were significantly different compared to those in the cDNA (plasma viral RNA loads, $p<0.01$; $CD4^+$ T cell numbers, $p<0.01$) and rDNA (plasma viral RNA loads, $p<0.01$; $CD4^+$ T cell numbers, $p<0.01$) groups.

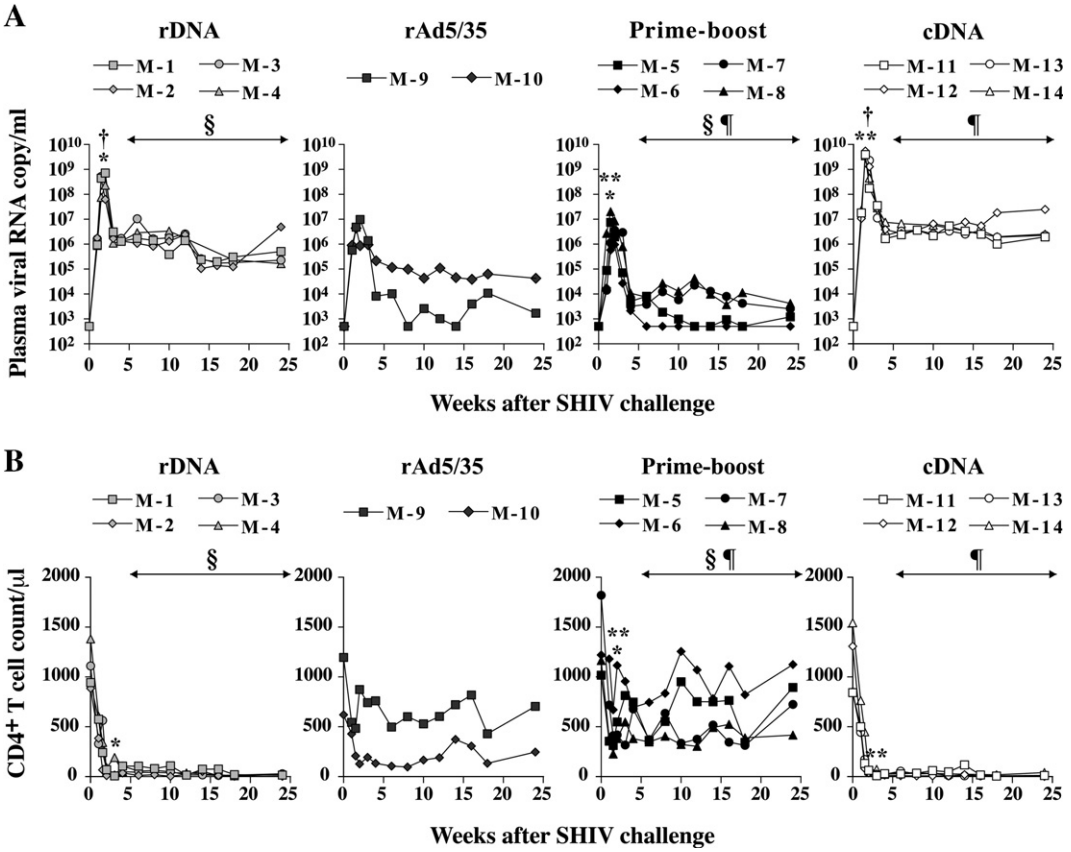

Fig. 2. Plasma viral RNA loads and $CD4^+$ T cell counts after challenge with SHIV. (A) Plasma RNA copies were determined by quantitative RT-PCR with a detection limit of 500 viral RNA copies per ml. *rDNA vs. prime-boost, $p<0.01$ (acute phase). **Prime-boost vs. cDNA, $p<0.01$ (acute phase). †rDNA vs. cDNA, $p<0.01$ (acute phase). §rDNA vs. prime-boost, $p<0.01$ (set-point phase). ¶Prime-boost vs. cDNA, $p<0.01$ (set-point phase). (B) Whole blood was stained with CD3 and CD4 antibodies. $CD4^+$ T cell numbers were determined using flow cytometry. *rDNA vs. prime-boost, $p<0.01$ (acute phase). **Prime-boost vs. cDNA, $p<0.01$ (acute phase). §rDNA vs. prime-boost, $p<0.01$ (set-point phase). ¶Prime-boost vs. cDNA, $p<0.01$ (set-point phase).

T cells immune response to Gag and Env after challenge with SHIV

At an early phase of infection (1 week after challenge), IFN-γ ELISPOT responses showed that the prime-boost group had higher numbers of Gag- and Env-specific SFC (Gag specific, an average of 2385; Env specific, an average of 420) than those of the rAd5/35 group (Gag specific, an average of 920; Env specific, an average of 270) (Fig. 2A). Furthermore, lower numbers of SFC were seen in the rDNA group (an average of 153 SFC specific for Gag; an average of 103 SFC specific for Env) than those in the prime-boost group (Gag, $p<0.01$; Env, $p<0.01$). The cDNA group showed no responses (≤ 30 SFC). Taken together, these results suggest that the prime-boost regimen elicited the highest frequency of cellular memory T cells in response to viral challenge.

Discussion

Higher plasma viral RNA loads and lower $CD4^+$ T cell numbers have been linked to disease progression in HIV-infected individuals (Patke et al., 2002; Mellors et al., 1996, 1997), suggesting that the generation of robust HIV-specific immunity may be critical in the control of viral infection. Recently, it was shown that a recombinant Ad5 vector-based HIV vaccine induced high levels of cellular immunity and protected animals from highly pathogenic viral challenge (Shiver et al., 2002). In contrast, other studies have shown that preexisting anti-Ad5 immunity can attenuate the efficacy of recombinant Ad5-based HIV vaccine (Barouch et al., 2004; Kostense et al., 2004; Casimiro et al., 2003). It is essential that such preexisting anti-Ad5 immunity be overcome if we are to enjoy the benefits offered by Ad5 as a vaccine vector, such as its induction of strong T cell immunity. Rare serotype Ad35, which is classified into subgroup B, has different cell tropism from Ad5 (Marttila et al., 2005; Segerman et al., 2000; Shayakhmetov et al., 2000; De Jong et al., 1997), and the seroprevalence of Ad35 in immunocompromised individuals and those at risk of AIDS was lower than that of Ad5, suggesting that Ad35 could be used as the base for an HIV vaccine (Kostense et al., 2004; Vogels et al., 2003). Therefore, the advantage of constructing a chimeric Ad5/35 vaccine is that the vector will retain its infectivity with less risk of preexisting immunity against the vaccine vector than that of Ad5-based vaccine. This suggests that the Ad5/35 vaccine would allow the basis for adenoviral vectors to be broadened with improved properties.

In this study, we initially constructed Ad5/35 vector-based HIV vaccine and demonstrated that its immunogenicity and protective efficacy in a macaque model. The rAd5/35 vector was previously developed as a vaccine vehicle for DC targeting (Xin et al., 2005, 2007; Mizuguchi and Hayakawa, 2002), and the rAd5/35 vector showed much lower hepatotoxicity than Ad5 (Xin et al., 2005). Recently, Ophorst et al. (2004) reported that rAd5 vector carrying a part of the fiber molecule of Ad35 (rAd5. Fib35) was less immunogenic in monkeys than rAd5, and rAd5. Fib35 showed no significant difference in anti-insert immunity over Ad5 in mice with high Ad5 vector-specific immunity. In addition, Abbink et al. (2007) reported comparative seroprevalence and immunogenicity studies involving rAd11, rAd35 and rAd50 vectors from subgroup B, rAd26, rAd48 and rAd49 vectors from subgroup D and rAd5 vectors from subgroup C. These rAd vectors were rare serotypes and elicited Gag-specific cellular immune responses in mice both with and without anti-Ad5 immunity. However, the rare serotype-based rAd vectors have proven less immunogenic than rAd5 vectors in animal models in spite of the absence of anti-Ad5 immunity (Barouch et al., 2004; Lemckert et al., 2005). In our previous study, rAd5/35 vector was highly immunogenic and significantly less susceptible to preexisting anti-Ad5 immunity than a comparable Ad5 vector (Xin et al., 2005). Although we did not compare the rAd5/35 vector directly with other serotype rAd vectors, we demonstrated that the rAd5/35 vector was immunogenic, and a much higher degree of immunogenicity was achieved by adopting a prime-boost regimen combining rAd5/35 vectors with rDNA vaccine in macaques.

To evaluate the efficacy of the vaccine, we monitored T cell responses, plasma viral RNA loads and $CD4^+$ T cell numbers following SHIV challenge. In the present study, we used highly pathogenic SHIV-C2/1 (Shinohara et al., 1998), which originated from SHIV 89.6 (Lu et al., 1996; Reimann et al., 1996) because our vaccine regimen was designed to target SIV Gag and HIV Env antigens and for the virus to well reproduce high plasma viremia and loss of $CD4^+$ cells (Eda et al., 2006; Someya et al., 2005, 2006; Ami et al., 2005; Shinohara et al., 1998).

After challenge with SHIV, all animals in the prime-boost and the rAd5/35 groups showed high memory T cell responses against Gag and Env, and these animals showed a reduction in peak viral RNA loads at the acute phase of infection. Furthermore, in the set-point phase, two animals in the prime-boost group and one animal in the rAd5/35 group controlled SHIV infection, and the remaining two animals in the prime-boost and one animal in the rAd5/35 groups showed moderate control. In spite of higher cellular responses being observed in the prime-boost group than those in the rAd5/35 group, the difference in protective levels between the prime-boost and the rAd5/35 groups was not clearly separated. Although it is not possible to directly compare the levels of immune responses and protective efficacy induced by the rAd5/35 vector to those of other rAd vector studies, our results appeared to be similar to those obtained with the other rAds (Abbink et al., 2007; Barratt-Boyes et al., 2006; Ophorst et al., 2004; Shiver et al., 2002; Musey et al., 1997). To clarify the efficacy of the rAd5/35 vector, further studies to compare with other rAd vectors may be needed. However, our results suggest that the rAd5/35 vector-based vaccine either alone or combined with DNA vaccine elicited a high frequency of viral-specific cellular T cell response that may well be key to controlling the acute and chronic phases of infection.

In conclusion, we have demonstrated that high doses of rAd5/35 vector-based vaccine afford protective immunity against SHIV in macaques. Since the rAd5/35 vector-based vaccine can bypass preexisting anti-Ad5 immunity, therAd5/35-based vaccine may offer considerable promise as an HIV vaccine.

Materials and methods

Animals

Fourteen adult cynomolgus macaques (*Macaca fascicularis*) were used in this study. The macaques were fed and cared for in accordance with the standard operating procedure approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan. The study was performed in the P3 facility under guidelines established by the laboratory biosafety manual of World Health Organization (Someya et al., 2005).

Preparation of DNA and recombinant adenoviral vectors

A eukaryotic expression plasmid, pcDNA3.1 (−) (Invitrogen, Carlsbad, CA), was used as a backbone of the DNA vaccines that encode the SIVmac239 *gag* gene (rDNA-Gag) and HIV-1 89.6 *env* gp160 gene (rDNA-Env), and both DNA vaccines were constructed as previously described according to the standard protocol (Someya et al., 2004). The SIV and HIV DNAs for the DNA vaccines were not modified using humanized codon. No foreign gene encoding pcDNA 3.1 (−) was used as a control DNA vaccine (cDNA).

The E1 and E3 regions deleted recombinant Ad5/35 vectors that encoded the SIVmac239 *gag* gene (rAd5/35-Gag) and HIV-1 89.6 *env* gp160 gene (rAd5/35-Env) were constructed with an Ad generation kit (Avior Therapeutics, Inc., Seattle, WA) as previously described (Xin et al., 2005; Mizuguchi and Hayakawa, 2002). Briefly, SIV *gag* and HIV *env* PCR fragments driven by a CAG promoter were inserted into a shuttle plasmid, pLHSP (Avior Therapeutics, Inc.), before being transfected to human embryonic kidney (HEK293) cells. The recombinant vectors were purified by CsCl gradient centrifugation, and the total concentration of virus particles was calculated from the optical density at 260 nm (OD_{260}), using the formula 1 $OD_{260} = 1 \times 10^{12}$ virus particles/ml.

Immunizations and viral challenge

Four animals of the rDNA group (numbered M-1 through M-4) received three intramuscular injections of both 2.5 mg of rDNA-Gag and 2.5 mg of rDNA-Env at 8-week intervals (Weeks 0, 8 and 16), followed by two intradermal injections of control Ad5/35 expressing the gene LacZ (cAd5/35, 2×10^{11} particles of each) at 2-week intervals (Weeks 150 and 152). Four animals of the prime-boost group (numbered M-5 through M-8) received three injections of both 2.5 mg of rDNA-Gag and 2.5 mg of rDNA-Env, followed by two injections with both 10^{11} particles of rAd5/35-Gag and 10^{11} particles of rAd5/35-Env. Two animals of the rAd5/35 group (numbered M-9 and M-10) received three injections of 5 mg of cDNA, followed by two injections with both 10^{11} particles of rAd5/35-Gag and 10^{11} particles of rAd5/35-Env. The four control animals of the control group (numbered M-11 through M-14) received three injections of 5 mg of cDNA, followed by two injections with 2×10^{11} particles of cAd5/35 (Table 1). All animals received an intravenous challenge with twenty 50% tissue culture infectious doses ($TCID_{50}$) of highly pathogenic SHIV that originated from SHIV-89.6 (Someya et al., 2006; Shinohara et al., 1998, Lu et al., 1996; Reimann et al., 1996) 2 weeks after final immunization (at 154 weeks, Table 1).

Cellular immune response to SIV Gag and HIV Env

The cellular immune responses against SIV Gag and HIV Env were monitored by IFN-γ ELISPOT assay using SIVmac239 Gag peptide pools spanning the full length of the Gag protein and HIV-89.6 Env V3 peptides as previously described (Someya et al., 2005, 2006). Freshly isolated peripheral blood mononuclear cells were added to 96-well plates in triplicate at 2×10^5 cells/well and then incubated for 16 h with Gag peptide pools or V3 peptide. Individual IFN-γ spot-forming cells (SFC) were counted by using a KS ELISPOT compact system (Carl Zeiss, Jena, Germany). An SFC was defined as a large black spot with a fuzzy border (Mothe et al., 2002).

Antibody responses to SIV Gag and HIV Env

The humoral immune responses were determined by measuring anti-Gag- and anti-Env-specific IgG titers using ELISA as previously described (Someya et al., 2005, 2006). Ninety-six-well ELISA plates were coated with 1 μg/ml of SIV p27 Gag (Advanced Biotechnologies, Advanced Biotechnologies, Woburn, MA) or 5 μg/ml of SHIV 89.6P Env peptide (AIDS Research and Reference Program, National Institutes of Health, Rockville, MD). Heat-inactivated sera were serially diluted and then added to the ELISA plate. Anti-Gag- and-Env-V3-specific IgG bound to the antigens were detected with alkaline phosphatase-labeled goat anti-monkey IgG (EY Laboratories, Inc., San Mateo, CA) and *p*-nitrophenyl-phosphate disodium substrate (Invitrogen, Carlsbad, CA).

The SHIV-specific neutralization antibody assay was performed as previously described (Someya et al., 2005, 2006). In brief, 5 μg/ml of purified serum IgG was incubated with 100 $TCID_{50}$ of SHIV-C2/1 and then cultured in M8166 cells. The result was compared with cultures to which preimmune IgG had been added. Neutralization was expressed as the percent inhibition of SIV Gag production in the culture supernatant. The average results + 6 SD of serum Ig from normal healthy monkeys were used as the cutoff for a positive titer (Someya et al., 2006).

Plasma viral RNA levels and $CD4^+$ T cell counts

SHIV RNA levels in plasma samples were determined by real-time PCR with a PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Forest City, CA) as previously described (Someya et al., 2006; Sasaki et al., 2002). All RNA samples were amplified in duplicate. Data were expressed as RNA copies per milliliter.

$CD4^+$ T cell numbers were measured using a FACS Calibur flow cytometer system (Becton-Dickinson Bioscience, San Jose, CA) as previously described (Someya et al., 2006; Yoshino et al., 2000). Fifty microliters of whole blood specimens was stained

with anti-human CD3 (clone HIT3a, Becton-Dickinson), anti-human CD4 (clone SK3 Becton-Dickinson) and anti-human CD8 (clone SK1, Becton-Dickinson). CD3/CD4 effective T cell counts were analyzed using Cell Quest software (Becton-Dickinson).

Statistical analysis

Comparisons of test results among groups of animals were performed using the Kruskal–Wallis *H*-test followed by Student–Newman–Keuls correction.

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